Invited Review

Thirty Years of Discovering Arthropod Alkaloids in Amphibian Skin†

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Amphibian skin has provided a wide range of biologically active alkaloids. During the past 30 years, over 400 alkaloids of over 20 structural classes have been detected. These include the batrachotoxins, which are potent and selective activators of sodium channels, the histrionicotoxins, which are potent noncompetitive blockers of nicotinic receptor-gated channels, the pumiliotoxins and related allo- and homo-pumiliotoxins, which have myotonic and cardiotonic activity due to effects on sodium channels, and epibatidine, which has potent antinociceptive activity due to agonist activity at nicotinic receptors. Further classes of alkaloids from amphibian skin include pyrrolidines and piperidines, decahydroquinolines, pyrrolizidines, various indolizidines, quinolizidines, and tricyclic gephyrotoxins, pyrrolizidine oximes, pseudophrynamines, coccinellines, and cyclopentaquinolizidines. Most alkaloids of amphibian skin appear to be sequestered from dietary arthropods. The source of the batrachotoxins, histrionicotoxins, pumiliotoxins, epibatidine, and certain izidines are unknown.

Amphibians have provided a remarkable array of biologically active compounds,[1] which on secretion from so-called granular skin glands can serve to protect the amphibian from predators due to noxious effects on buccal tissue and, at least in the case of some peptides, to protect from bacterial or protozoan infections.

A wide range of peptides and biogenic amines have been discovered in amphibian skin, beginning with the pioneering work of Vittorio Erspamer,[2] who has discovered many new classes of peptides, many of which have mammalian roles as hormones or neuromodulatory agents. The peptides and biogenic amines are synthesized by the amphibian, and their high levels must be due to overexpression of pathways responsible for their formation and storage. In recent times, research has focused on antimicrobial peptides, initiated by the discovery of the amphiphilic magainins by Michael Zasloff.[3] One such amphiphilic peptide, adenoregulin, was discovered by our group in an investigation of secretions of a tree frog used to attain "hunting magic" powers by certain Amazonian Indians.[4] Adenoregulin affects receptor-G-protein coupling apparently by enhancing GTPase activity of the G-proteins.[5]

A wide range of steroidal bufadienolides have been discovered, beginning with the pioneering work of Heinrich Wieland and later Klaus Meyer on parotoid glands of bufonid toads of the genus *Bufo*.[6] Skin from bufonid toads of the genera *Atelopus*, *Dendrophryniscus*, and *Melanophryniscus* have also been found by our group to contain bufadienolides or related compounds, on the basis of assays of inhibition of Na^+/K^+-ATPase or binding of tritiated ouabain.[7] Bufonid toads of the genera *Bufo* and *Atelopus* synthesize and store such noxious and toxic steroids, based both on biosynthetic studies[8] and on production in captive-raised specimens.[9]

Another class of amphibian toxins are the tetrodotoxins, which were originally discovered in a newt of the genus *Taricha* by Harry S. Mosher.[10] Mosher also discovered two tetrodotoxin-like water-soluble alkaloids, zetekitoxin and chiriquitoxin, in bufonid toads of the genus *Atelopus*.[11] The structure of chiriquitoxin has been elucidated,[12] while that of zetekitoxin will probably never be revealed because of overprotection of the Panamanian species *Atelopus zeteki* that produces it. Captive-raised bufonid toads of the genus *Atelopus* do not contain any tetrodotoxin in their skin[9] consonant with proposals for an origin of tetrodotoxins in higher organisms from symbiotic microorganisms. Tetrodotoxins have also been found by our group in one species, *Colostethus inguinalis*, of the family Dendrobatidae.[13]

Our initial work focused for nearly 20 years on the noxious and in some cases quite toxic lipophilic alkaloids that we found in the skin of neotropical frogs of the family Dendrobatidae. When we began, there was already a precedent for "animal alkaloids" in the work of Clement Schöpf on the steroidal samandarines of the European fire salamander, *Salamandra salamandra*.[14] My own involvement with dendrobatid frogs began in 1963 when my Laboratory Chief Bernard Witkop approached me to propose that I become involved in what was considered dangerous field work on a frog used by Indians of the Western coast of Colombia to poison blow darts. The active principles had been found by a

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postdoctorate, Fritz Märki, in our laboratory to be highly toxic alkaloids,[15] and it was clear that collection of sufficient frogs and separation and structure elucidation of active principles would be a major challenge. I embarked on my first of several collecting trips to the Río San Juan in Western Colombia in January 1964 and was successful in obtaining several thousand specimens of the poison-dart frog *Phyllobates aurotaenia*, from which four toxic alkaloids were isolated from the methanol extracts by classical solvent partition and column chromatography. The three major alkaloids after final structure elucidation were to be named batrachotoxin, homobatrachotoxin, and batrachotoxinin A.[16] The fourth pseudobatrachotoxin was unstable and converted on standing to batrachotoxinin A. Isolation of batrachotoxins was initially followed by a sensitive bioassay based on toxicity. Only 200 ng was sufficient on subcutaneous injection to kill a mouse in 8 min. It was soon discovered that the two most toxic alkaloids, batrachotoxin and homobatrachotoxin, could be assayed by a modified Ehrlich reagent with a detection limit of less that 50 ng. The positive Ehrlich reaction suggested the presence of a pyrrole moiety, but our failure to detect the true molecular ion of batrachotoxin led us to surmise from an apparent molecular ion at mass 399 ($C_{24}H_{33}NO_4$) that batrachotoxin was a steroidal alkaloid, whose single basic nitrogen was converted under conditions of the Ehrlich test into the nonbasic nitrogen of a pyrrole moiety. This was in the late 1960s before the advent of chemical ionization, fast-atom bombardment, and other powerful mass spectral techniques. It was also before the advent of current powerful NMR spectral techniques that would have facilitated interpretation of the NMR spectra. It was determined that the solution to the structures would be to prepare a crystalline derivative of batrachotoxinin A, the most abundant, and also least toxic of the three major alkaloids. Batrachotoxinin A did not give an Ehrlich reaction, and with a molecular ion at mass 417 was at first thought to be a "hydrate" of batrachotoxin. Takashi Tokuyama had begun working with me and he succeeded in 1968 in obtaining the 20α-*p*-bromobenzoate of batrachotoxinin A in crystalline form. Isabella Karle determined the structure, and we then realized from our accumulated spectral data that batrachotoxin and homobatrachotoxin were (20α)-dimethylpyrrole-3-carboxylates of batrachotoxinin A. Tokuyama made all of the possible isomeric ethyl dimethylpyrrole-3-carboxylates and showed by comparison of solvent-induced shifts in the NMR methyl resonances that batrachotoxin was the (20α)-2,4-dimethylpyrrole-3-carboxylate and homobatrachotoxin the (20α)-2-ethyl-4-methylpyrrole-3-carboxylate[16] (Figure 1). The structure of batrachotoxin was confirmed by partial synthesis from the (20α)-alcohol batrachotoxinin A, and the true molecular ion was transiently found using an overloaded direct probe at mass 538 as expected. Dr. Tokuyama continued to work with me over the years on the dendrobatid alkaloids, both during his sojourn at NIH and after his return to Japan.

Pharmacological studies on batrachotoxin in collaboration with Edson X. Albuquerque defined its target as a site on the voltage-dependent sodium channel of nerve and muscle.[17] A radioligand, the tritiated (20α)-benzoate of batrachotoxinin A, was developed.[18] Batrachotoxin and its radioactive analogue became important tools in our understanding of the allosteric control of sodium channel function by local anesthetics, anticonvulsants, antiarrythmitics, and various toxins. The toxins include pyrethroids, the brevetoxins, and certain anenome and scorpion toxins.

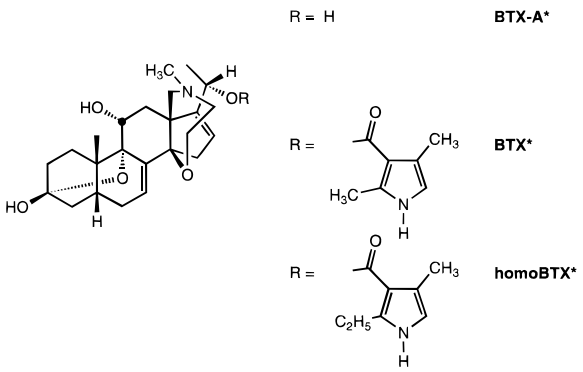


Figure 1. Structures of batrachotoxin (BTX), homobatrachotoxin (homoBTX), and batrachotoxinin-A (BTX-A). *Absolute configuration known.

The steroidal structure of the batrachotoxins was unique, and related alkaloids were unknown in nature. However, in 1990, I was contacted by an ornithologist, Jack Dumbacher, who was doing his graduate work in Papua New Guinea and had recognized on handling certain passerine birds of the genus *Pitohui* that there were unpleasant, perhaps toxic, principles in the feathers. He sent feathers, skin, muscle and internal organs in alcohol to the NIH. The extracts from skin and feathers were toxic and proved to contain trace amounts of a single highly toxic alkaloid. A mass spectra of the trace alkaloid isolated by thin-layer chromatography immediately identified it as homobatrachotoxin,[19] whose mass spectral properties I had labored over in the late 1960s.

Over the years before the discovery of homobatrachotoxin in feathers and skin of a passerine bird, we had discovered and characterized hundreds of alkaloids in skin of four genera of frogs of the family Dendrobatidae[1] (see Figure 2). The batrachotoxins occurred only in the skin of the five frogs of the monophyletic dendrobatid genus *Phyllobates*, while a variety of simpler lipophilic alkaloids occurred in the skin of frogs of the dendrobatid genera *Dendrobates*, *Epipedobates*, and *Minyobates*. No lipophilic alkaloids were found in skin of the remaining two dendrobatid genera *Colostethus* and *Aromabates*. Over the years, no alkaloids were found in skin from frogs/toads of nearly 70 other genera from 11 families of anurans. Then, in the early 1980s, we discovered the so-called "dendrobatid alkaloids" in one genus each of frogs/toads from three other families[20] (see Figure 2). These were bufonid toads of the genus *Melanophryniscus* from subtropical southeastern South America, mantelline frogs of the genus *Mantella* from Madagascar, and myobatrachid frogs of the genus *Pseudophryne* from Australia. Except for the nocturnal *Pseudophryne*, all were primarily terrestrial, diurnal, small frogs/toads, ranging from 15 to 50 mm in snout to vent length. The majority of compounds, now numbering over 400,[21] were unknown elsewhere in nature, and we assumed that such alkaloids, occurring in genera from four different amphibian families, were being

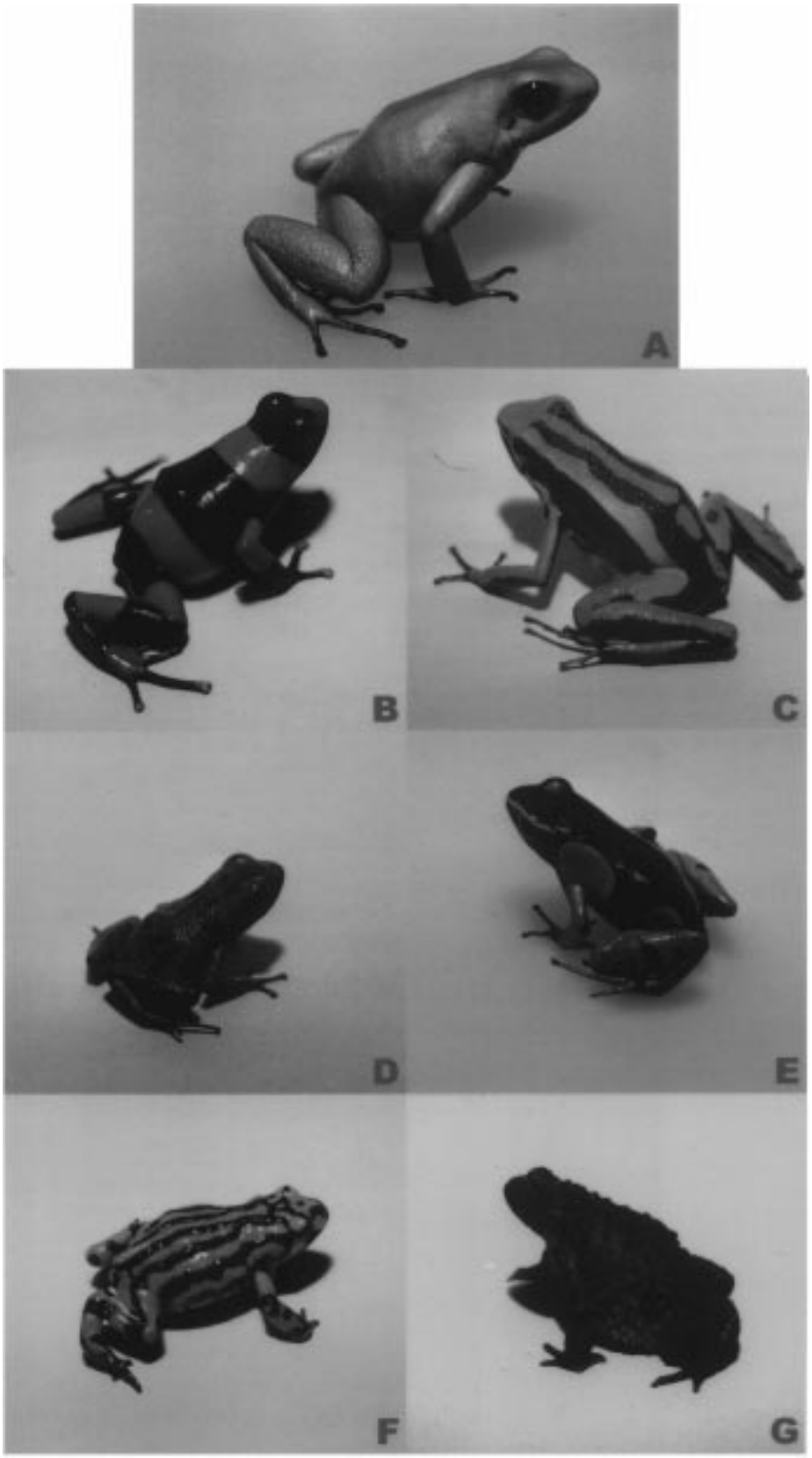


Figure 2. Representative anurans from the seven genera that contain lipophilic skin alkaloids that appear to be accumulated unchanged from dietary sources. (A) Colombian *Phyllobates terribilis* (Dendrobatidae) containing batrachotoxins. (B) Colombian *Dendrobates lehmanni* (Dendrobatidae) containing a unique 1-azabicyclo[5.3.0]decane. (C) Peruvian *Epipedobates trivittatus* (Dendrobatidae) containing mainly histrionicotoxins. (D) Venezuelan *Minyobates steyermarki* (Dendrobatidae) containing pumiliotoxins and a pyrrolizidine. (E) Madagascan *Mantella baroni* (Mantellinae) containing pumiliotoxins and izidines. (F) Australian *Pseudophryne corroboree* (Myobatrachidae) containing pumiliotoxins and pseudophrynamines. (G) Brazilian *Melanophryniscus moreirae* (Bufonidae) containing pumiliotoxins.

synthesized *de novo* from precursors by the frogs and hence would be potentially useful as chemotaxonomic features. But in the past few years we have been forced to the conclusion that the frogs do not make such alkaloids, but instead have developed or overexpressed systems that serve to accumulate alkaloids from dietary sources, which for dendrobatid frogs consist of small and even tiny arthropods, such as mites, ants, springtails, and flies, unchanged into the granular poison glands of their skin. The identity of the dietary arthropods that supply the batrachotoxins, pumiliotoxins, histrionicotoxins, epibatidine, and several other classes of alkaloids remain a complete mystery despite our efforts over the past 5 years. We have shown that six classes of alkaloids undoubtedly come from dietary ants, one class from beetles, and one class from small millipedes,[22−24] but the sources of the alkaloids that are the most interesting, both structurally and from the standpoint of biological activity, remain shrouded in mystery. Thus, the ultimate arthropod sources may provide a further treasure-trove of alkaloids that might never have been discovered if the frogs had not targeted such arthropods as a source of alkaloids to serve in their own defense. In addition to developing active alkaloid-sequestering systems, frogs of the genus *Phyllobates* have evolved a modified sodium channel that no longer responds to the batrachotoxins,[25] thus allowing them to eat with impunity the mysterious and undoubtedly highly toxic batrachotoxin-containing arthropod. Most of the other dendrobatid alkaloids are not nearly as toxic as the batrachotoxins and thus probably would not deter the poison frogs from targeting dietary prey containing such alkaloids. Whether the homobatrachotoxin found in *Pitohui* birds has a dietary source or is formed *de novo* is unknown.

Our research at the NIH on further alkaloids from frog skins might have ended with batrachotoxin were it not for a news release that appeared in 1966 in *Medical World News* on our work on batrachotoxin from the Colombian poison-dart frog. This news release drew the attention of a herpetologist, Charles W. Myers, at that time in Panama as a graduate student. He wrote to me suggesting a collaboration on a small dendrobatid frog, *Dendrobates pumilio*, in particular, the possible correlations of coloration, behavior, and toxicity among the very distinct populations of this frog found in the Bocas archipelago of Panama. I replied that I was enthusiastic about such a collaboration and we planned logistics for field work. My enthusiasm was due to prior data obtained in Colombia that indicated that further, novel alkaloids would be present in other dendrobatid species, such as *Dendrobates histrionicus*, a species that occurred sympatric with the batrachotoxin-containing *P. aurotaenia* at our collection site on the upper Río San Juan. This frog did not contain batrachotoxins, but did contain lower molecular weight alkaloids with distinctive mass spectra. Thus began a life-long friendship and collaboration with Charles W. Myers, currently head of the Department of Herpetology at the prestigious American Museum of Natural History in New York. The initial studies began in the archipelago of Bocas in Panama. The results did not reveal any correlations among brightness of coloration, behavior, and toxicity[26] but did suggest, based on TLC analysis, that different profiles of alkaloids would be found in different populations of one species of dendrobatid frog. A GC system for quantitating and, when combined with MS, for characterizing the mixture of alkaloids present in different skin extracts, was established. Fortunately, most of the alkaloids proved amenable to such GC analysis. GC analytical techniques have advanced greatly since those pioneering days and we now can characterize by EI-MS and CI-MS, both before and after deuterium exchange with ND_3, the many individual alkaloids often found in extracts from a single frog skin. Recently, we have introduced the technique of NH_3-CI-MS/MS analysis of such alkaloids.[27] We have found that the ionic fragmentations of the protonated parent ion (CI-MS/MS) are remarkably different from the fragmentations of the cationic free-radical parent ion (EI-MS). In particular, the dominant α-cleavages proximal to nitrogen of EI-MS spectra are not seen in NH_3-CI MS/MS spectra. In addition to MS analyses, GC FTIR spectral analyses now provide further structural insights, particularly with respect to functional groups and stereochemistry. Finally, NMR techniques have advanced during the 1980s and into the 1990s to the point where structure definition of the alkaloids from frog skin can be done almost routinely on 500 μg of pure compound isolated by HPLC.

The initial studies in Bocas, Panama, did lead to isolation of two toxic alkaloids from 20 skins of an abundant population of *Dendrobates pumilio* found on Isla Bastimentos.[26] Further characterization with material from subsequent fieldwork led to the conclusion that the two alkaloids pumiliotoxin A and B were bicyclic alkaloids with two double bonds and a side chain terminating in $-CHOHCH_2CH_3$ and $-CHOHCHOHCH_3$, respectively. Analyses of the NMR spectra of the two pumiliotoxins done in the early 1970s did not define the bicyclic ring system. Efforts to prepare crystals were frustrated by the instability of pumiliotoxin A and B apparently because of an allylic hydroxyl group. The MS with major fragment ions at mass 166 and 70 were diagnostic for such alkaloids of which over 20 have now been detected. Thus, in subsequent field work, Myers and I sought a species or population of frogs that would contain sufficient amounts of a simpler pumiliotoxin for crystallization and X-ray analysis. By serendipity, we obtained such a frog in 1974. At that time, we did not realize that skin extracts from the frog *Epipedobates tricolor* from near Santa Isabel in southwestern Ecuador contained major amounts of a simpler pumiliotoxin, which because of its volatility had been lost during rotary evaporations of the methanolic extracts. Luckily, there was another alkaloid, present in only trace amounts in that extract, which caused in mice the Straub-tail reaction that is typical of opioid alkaloids. Because of the Straub-tail alkaloid, 750 skins were obtained in a subsequent field trip with Myers in 1976, which yielded both the Straub-tail alkaloid in amounts of less than 1 mg and 21 mg of pumiliotoxin **251D**. The latter was crystallized as the hydrochloride salt, and X-ray analysis provided not only its structure, but the key to the structures of pumiliotoxin A and B and related allopumiliotoxins[28] (Figure 3).

A numbering system for "dendrobatid alkaloids" had been introduced in 1978[29] because of the large number

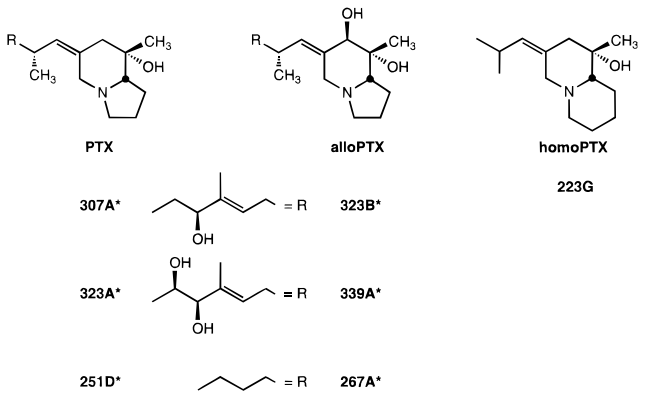


Figure 3. Structures of pumiliotoxin A (**307A**) pumiliotoxin B (**323A**), pumiliotoxin **251D**, allopumiliotoxins **323B**, **339A**, and **267A**, and homopumiliotoxin **223G**. *Absolute configuration known.

of alkaloids being discovered. It consisted of a boldface number corresponding to the nominal mass and a boldface letter corresponding to different alkaloids with the same nominal mass. The system became less satisfactory when capillary gas chromatography began to reveal different stereoisomers of the "dendrobatid alkaloids", and *cis*, *trans*, *epi*, *iso*, and other notations have been used to designate such stereoisomers, when structures have been spectrally defined. The pumiliotoxin class now consists of over 30 compounds, which are subdivided into pumiliotoxin and allopumiliotoxin subclasses. The allopumiliotoxins have a 7-hydroxy substituent and hence give a major fragment ion at mass 182 instead of the mass 166 fragment of the pumiliotoxins. The pumiliotoxins and allopumiliotoxins occur in all genera of frogs/toads that have lipophilic alkaloids in skin, and hence, the unknown dietary source must have a wide distribution.

Homopumiliotoxins, a closely related class of alkaloids with a quinolizidine instead of an indolizidine ring have also been discovered.[30] Such alkaloids have major fragment ions at mass 180 and 84. Only one, homopumiliotoxin **223G**, has been isolated in sufficient quanity for NMR analysis, and it is also shown in Figure 3.

Confirmation of the proposed structures of pumiliotoxins and allopumiliotoxins has been largely due to the synthetic efforts of Larry Overman.[31] Collaborative studies with Overman using synthetic pumiliotoxins and allopumiliotoxins allowed definition of structure–activity relationships for these cardiotonic and myotonic alkaloids.[32] At least three hydroxyl groups appear requisite for high cardiotonic activity, two of which can be contributed either by the side chain as in pumiliotoxin B (**323A**) or by the ring as in allopumiliotoxin **323B**. Absence of a side-chain hydroxyl appears to eliminate cardiotonic activity. The primary site of action of pumiliotoxin B was defined by Fabian Gusovsky of my group as being a subdomain of the batrachotoxin-modulatory site on the voltage-dependent sodium channel.[33,34] A radioligand has not yet been developed, and further research on the potential of such alkaloids as cardiotonic and myotonic agents is needed.

The third alkaloid isolated in those first studies in Panama on *D. pumilio* was referred to as pumiliotoxin C.[35] It is virtually nontoxic, and to avoid confusion with the pumiliotoxin class, it is now referred to as decahydroquinoline *cis*-**195A**. It crystallized readily, and X-ray

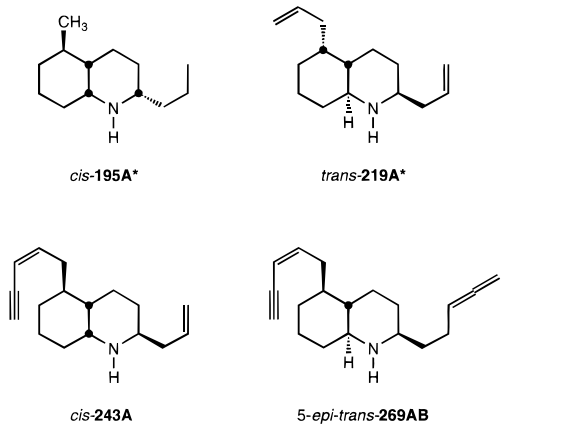


Figure 4. Structures of decahydroquinolines *cis*-**195A**, *trans*-**219A**, *cis*-**243A**, and 5-*epi*-*trans*-**269AB**. *Absolute configuration known.

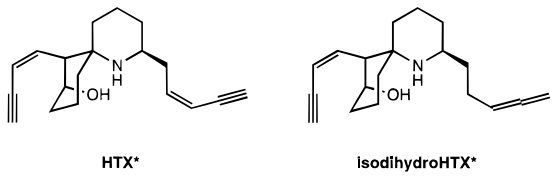


Figure 5. Structures of histrionicotoxin (**283A**) and isodihydrohistrionicotoxin (**285A**). *Absolute configuration known.

analysis provided the structure, which is shown along with other decahydroquinolines in Figure 4. The structure of *trans*-**219A** was also established by X-ray analysis and differs in absolute stereochemistry at all but one of the four chiral carbons. Some 30 or more *cis*- and *trans*-decahydroquinolines have now been discovered in skin extracts of frogs. Several have been isolated in sufficient quantities for NMR analysis. Analysis of vapor-phase FTIR bands in the fingerprint region can define the *cis*- or *trans*- nature of the ring fusion, while analysis of Bohlmann bands provides for assignment of stereochemistry of hydrogens on carbons attached to nitrogen in decahydroquinolines and also in pyrrolidines, piperidines, and izidines.[36,37] Decahydroquinolines have now been discovered by our group in collaboration with Tappey H. Jones in myrmicine ants.[24,38]

After the initial field work in the Bocas region of Panama, Myers and I determined that we should undertake a complete survey of alkaloids in all species of dendrobatid frogs. This led not only to characterization of hundreds of alkaloids, but to discovery of several new dendrobatid species. Our first memorable field work together allowed us to sample many Colombian populations of *Dendrobates histrionicus*, an extremely variable species, that contained as their major alkaloids not batrachotoxins or pumiliotoxins, but instead what we were to term histrionicotoxins. Like the decahydroquinolines, the histrionicotoxins are virtually nontoxic and the name is a misnomer. From extracts of skin from an extremely abundant population of *D. histrionicus* at the pueblo of Guayacana near the Colombian border with Ecuador, we isolated three major alkaloids, two of which crystallized readily, and structures were defined as histrionicotoxin and isodihydrohistrionicotoxin (Figure 5).[39] The acetylenic and allenic bonds and the spiropiperidinol ring system are remarkable. Histrionicotoxins have been found only in dendrobatid

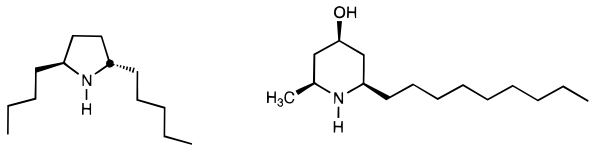


Figure 6. Structures of monocyclic alkaloids: pyrrolidine **197B** and piperidine **241D**. *Absolute configuration known.

frogs of Central and South America. They do not occur in a dendrobatid frog introduced into Hawaii, nor in mantelline, myobatrachid, and bufonid species.[22,23] Some 16 histrionicotoxins have been characterized. Like the decahydroquinolines, histrionicotoxins with 15, 17, and 19 carbons are found in dendrobatid frogs, and often the 19-carbon decahydroquinolines occur in consort with the 19-carbon histrionicotoxins. Both classes of alkaloids can have diene, acetylene, and allene bonds in their side chains. It is possible that, like the decahydroquinolines, the histrionicotoxins derive from a dietary ant source.

The histrionicotoxins were shown in collaboration with Edson X. Albuquerque to be potent noncompetitive blockers of neuromuscular nicotinic channels.[40,41] The blockade was stimulus-dependent, indicating open-channel blockade. Later studies demonstrated that histrionicotoxins also block ganglionic and central neuronal nicotinic channels.[42] Tritiated perhydrohistrionicotoxin was developed and used as a radioligand for the study of structure−activity relationships for binding to a site on neuromuscular-type nicotinic channels of the *Torpedo* electroplax.[43] In addition to phencyclidine, quinacrine, local anesthetics, chlorpromazine, and other drugs, many of the frog skin alkaloids, including decahydroquinolines, pyrrolidines, piperidines, izidines, and gephyrotoxins, have proven to be noncompetitive blockers of nicotinic channels.[42]

During the 1970s, Myers and I were to make many more field trips to collect dendrobatid frogs in Costa Rica, Panama, Colombia, Ecuador, Peru, Venezula, and Surinam and to prepare skin extracts for analysis at the NIH. A range of relatively simple monocyclic pyrrolidines and piperidines, bicylic izidines, and tricyclic alkaloids were discovered (Figures6−8). The pyrrolidines were exemplified by **197B** and the piperidines by **241D**. Other pyrrolidines and piperidines were found, but only in trace amounts. The izidine alkaloids included 3,5-disubstituted pyrrolizidines, such as **223H** and **251K**, 3,5-disubstituted indolizidines, such as **195B** and **223AB**, 5,8-disubstituted indolizidines, such as **207A**, 5,6,8-trisubstituted indolizidines, such as **223A**, and 1,4-disubstituted quinolizidines, such as **217A**. Sufficient quantities were isolated for NMR analyses of many of the izidines. The MS fragmentation patterns were diagnostic, as were the Bohlmann bands of vapor-phase FTIR spectra.[37,44,45] The monocyclic pyrrolidines and piperidines and the bicyclic izidine alkaloids of dendrobatid frog skin may all prove to be derived from ants, but as yet no 5,8-disubstituted indolizidines, nor 5,6,8-trisubstituted indolizidines, nor 1,4-disubstituted quinolizidines have been detected from ants. Recently, the structure of a 4,6-disubstituted quinolizidine **195C**, which occurs in both frog skins and in an ant, has been determined in collaborative studies with Tappey H. Jones.[24]

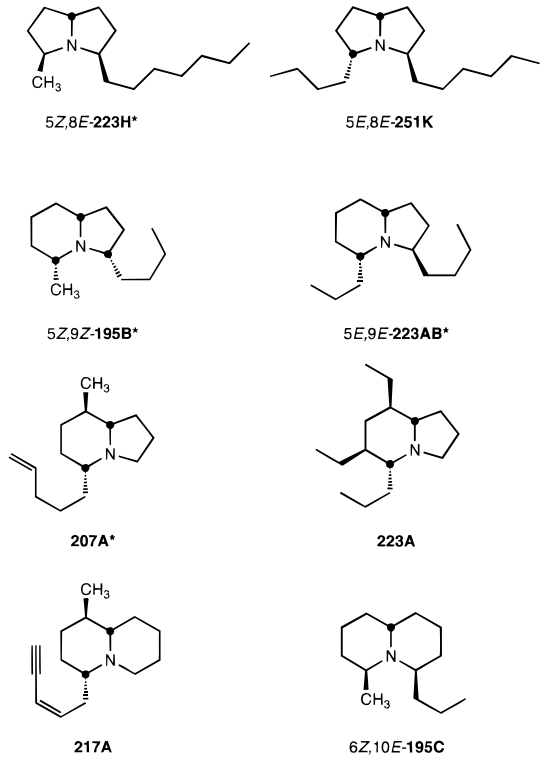


Figure 7. Structures of representative bicyclic izidine alkaloids: 3,5-disubstituted pyrrolizidine **223H** and **251K**, 3,5-disubstituted indolizidines **195B** and **223AB**, 5,8-disubstituted indolizidine **207A**, 5,6,8-trisubstituted indolizidine **223A**, 1,4-disubstituted quinolizidine **217A**, and 4,6-disubstituted quinolizidine **195C**. *Absolute configuration known.

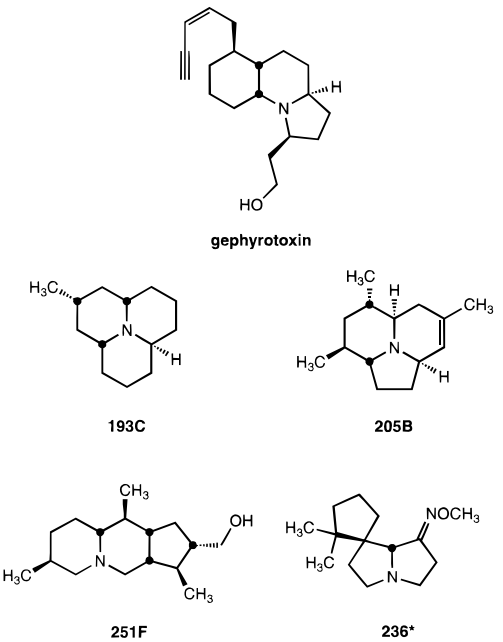


Figure 8. Structures of tricyclic alkaloids: gephyrotoxin (**287C**), precocinelline (**193C**), alkaloid **205B**, perhydrobenzoquinolizidine **251F** and spiropyrrolizidine **236**. *Absolute configuration known.

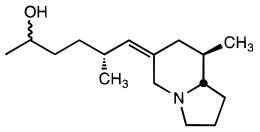
One izidine alkaloid was found in the 1970s as a unique alkaloid in frogs of unknown origin, which were sold in the pet trade both in streets of Cali and in Europe, and were considered to represent a striking red and black-banded population of *D. histrionicus*. The frog did not contain any histrionicotoxins, in marked

contrast to all populations of *D. histrionicus*, all of which contained histrionicotoxins as major alkaloids. After 2 years, Myers and I located the source, a montane cloud forest near the city of Cali. The unique profile of skin alkaloids was one factor that led us to define this frog as a separate new species, *Dendrobates lehmanni*. The izidine alkaloid **275A**, which occurs in significant amounts only in populations of *D. lehmanni*, proved a structural challenge. Very small amounts were finally isolated for NMR analysis. The combined MS and NMR data suggested that it was a 4-methyl-6-nonynylquinolizidine. Indeed, the perhydro derivative of **275A** was virtually identical with one of the four possible stereoisomers of a mixture of synthetic 4-methyl-6-nonylquinolizidines prepared by Tappey H. Jones, both in EI- and ion-trap-MS and in vapor-phase FTIR spectra (unpublished data). But there was a slight difference in GC retention times on a capillary column. The structure was solved by use of NH_3-CI-MS/MS. Both the perhydro derivative of **275A** and the 4-methyl-6-nonylquinolizidines prepared in this collaboration by Tappey H. Jones afforded virtually identical EI-MS spectra, consisting of α-cleavages to yield a minor fragment ion due to loss of methyl and a major fragment ion at mass 152 (base peak), due to loss of the nonyl side chain. However, the NH_3-CI-MS/MS spectra were remarkably different with the synthetic 4,6-disubstituted quinolizidines affording diagnostic fragments at mass 98 (base peak), representing the six-membered ring bearing the methyl, and at mass 210 representing the six-membered ring bearing the nonyl side chain. In the case of perhydro-**275A**, the NH_3-CI-MS/MS spectrum revealed a base peak at mass 112, indicating the methyl was on a seven-membered ring, and a peak at mass 190, indicating that the nonyl side chain was on a five-membered ring. Thus, the unknown alkaloid **275A** was a 4-methyl-9-nonynyl-1-azabicyclo[5.3.0]-decane, as has now been confirmed with synthetic diastereomers synthesized by Tappey H. Jones (unpublished data).

The tricyclic alkaloids found in the skin of dendrobatid frogs[21] include gephyrotoxin, which was isolated along with histrionicotoxin and isodihydrohistrionicotoxin from the abundant population of *D. histrionicus* found near Guayacana, Colombia.[39] Structure elucidation of this labile alkaloid, originally termed HTX-D, required X-ray analysis.[46] The structure of this 19-carbon alkaloid (Figure 8) proved reminiscent of the decahydroquinolines, as was the highly unsaturated side chain, which also is present in 19-carbon decahydroquinolines and histrionicotoxins. Gephyrotoxins are virtually nontoxic and occur only rarely in dendrobatid frogs and always with histrionicotoxins. Other tricyclics from dendrobatid frog skin (Figure 8) include the beetle alkaloid precocinelline (**193A**) and a tricyclic analogue, alkaloid **205B**. The original proposed structure of **205B**[30] has been slightly modified on the basis of further NMR and vapor-phase FTIR analyses (unpublished data). A tricyclic alkaloid **251F** occurs almost uniquely in skin extracts from a tiny dendrobatid frog, *Minyobates bombetes*, which was discovered in two strikingly disimilar habitats outside of Cali, Colombia. Some 300 μg was isolated from extracts of 100 frogs. The structure, on the basis of MS, FTIR, and NMR spectral analysis, is as shown in Figure 8.[47] Another tricyclic alkaloid was remarkable in the fact that it was not present in skin extracts from collections made in the 1960s and 1970s of a population of *D. pumilio* on Isla Bastimentos, Panama, but had become a significant alkaloid component along with two congeners in skin extracts from collections of the same population made in 1987. The spiropyrrolizidine oxime structure of this alkaloid **236** was proposed in 1992,[48] as a correction of a previously proposed amidine structure,[30] and was later confirmed in our group by synthesis.[49] The ring system is identical with that of nitropolyzonamine, an alkaloid isolated by Jerrold Meinwald and co-workers from a small North American millipede,[50] and its sudden emergence in frog skin after a 10-year hiatus in collection of *D. pumilio* on Isla Bastimentos undoubtedly is due to an increase in the abundance of such a millipede. Spiropyrrolizidine **236** is a noncompetitive blocker of nicotinic channels with selectivity toward a ganglionic subtype.

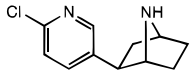
Further alkaloids isolated by HPLC from frog skin are currently being characterized. One is the 8-deoxypumiliotoxin **251H** (Figure 8),[51] isolated along with pumiliotoxin **251A** and the Straub-tail alkaloid from the extracts of *E. tricolor*, obtained in 1976. A unique cyclic ether of the pumiliotoxin class, namely **341A**, was also isolated from the same extracts, and its structure will soon be reported. The structures of both these alkaloids and several others have had to wait until the power and sensitivity of NMR spectrometry advanced to the stage that it was worthwhile to isolate by HPLC the submilligram quanities of such alkaloids present in complex mixtures often containing dozens of alkaloids.

The Straub-tail alkaloid also represents a striking example of how technical advances in instrumentation have made possible what was impossible 15 years ago. The Straub-tail alkaloid was discovered in skin extracts obtained from two Ecuadoran populations of a dendrobatid frog now considered, despite marked differences in size and appearance at the lowland and highland extremes of its range, to represent a single species, *E. tricolor*. Extracts from several specimens, which were collected on an exploratory trip to the west coast of Ecuador in 1974 from a cacao grove in the lowlands and from a road-side seepage area in the highlands, caused a marked Straub-tail reaction in mice. The alkaloid causing this Straub-tail reaction appeared to be present in trace amounts, and hundreds of frogs were deemed necessary for any hope of determining the structure. A second collecting trip was organized for 1976, but to our disappointment Myers and I found that the lowland population from the cacao grove had inexplicably vanished. Seemingly identical frogs were abundant in nearby banana plantations and a large collection was made, but to our surprise skin extracts from those frogs contained no alkaloids at all. Fortunately, the frogs were still abundant at the road-side highland site. Extract from a total of 750 frogs provided, in addition to pumiliotoxins, a total of nearly 1 mg of the Straub-tail alkaloid, which we were to ultimately name epibatidine. Analysis indicated that epibatidine was a basic, relatively polar alkaloid with an empirical formula of $C_{10}H_{13}N_2Cl$. A secondary nitrogen that could be acylated was present. The UV spectra and other data



8-deoxyPTX **251H**

Figure 9. Structure of 8-deoxypumiliotoxin **251H**.



epibatidine*

Figure 10. Structure of epibatidine. *Absolute configuration known.

strongly suggested the presence of a chloropyridine moiety, but NMR and MS spectral analysis in 1980 on such a small amount of material did not provide sufficient information to propose a structure. Efforts in 1982 to obtain more epibatidine were not successful due to changes in the road system at the highland site in Ecuador. Extracts from several hundred frogs from a nearby stream-side locale did contain epibatidine, but not in sufficient amounts to warrant isolation. Then, in 1984, the Convention on International Trade in Endangered Species (CITES) put all the dendrobatid frogs on their Appendix II listing. From a scientist's standpoint, such a CITES listing means that no investigator will ever be able to obtain permits to collect the requisite hundred or more specimens required for structure elucidation of minor and trace components found in dendrobatid frogs, even though such frogs are often incredibly abundant. Thus, in the mid-1980s, we had what had become an irreplaceable sample of epibatidine. We had used some sample to demonstrate that epibatidine was 200-fold more potent than morphine as an analgetic and that, unlike morphine, the Straub-tail and analgesia were not blocked by an opioid receptor antagonist, naloxone. In 1990, the power and sensitivity of NMR spectroscopy had advanced remarkably, and H. Martin Garraffo, Thomas F. Spande, and I discussed the feasibility of now defining by NMR analysis the structure of epibatidine. After pooling the fractions containing epibatidine, we realized that further purification would be needed prior to NMR analysis to remove some neutral contaminants and some pumiliotoxins. Further HPLC purification appeared too risky, and Garraffo suggested a potentially quantitative, solvent-partition separation with the epibatidine being converted to a weakly basic *N*-acetyl derivative, followed by removal with acid extraction of the highly basic pumiliotoxins, which as tertiary amines would not have been acetylated. On a 5-μg scale, a quantitative and complete purification of epibatidine as the *N*-acetyl derivative was accomplished. Nearly all of the epibatidine sample was now acetylated, and NMR analysis of the purified *N*-acetylepibatidine provided an unambiguous structure (Figure 10), which we then reported.[52]

We and many other laboratories then embarked on the synthesis of this remarkably potent analgetic. In May 1993, E. J. Corey called to inform me that they had succeeded in synthesizing both enantiomers of epibatidine and three analogues in which the chlorine atom of epibatidine had been replaced by hydrogen, methyl, or iodine. He inquired as to whether we would be interested in investigating the site of action of epibatidine, which we already suspected would be the nicotinic receptor-channel. Corey provided the five compounds, and we found in a binding assay and in three functional assays that epibatidine was the most powerful nicotinic agonist known.[53] Remarkably, there was no enantioselectivity. Replacement of the chlorine with hydrogen had virtually no effect on activity, while replacement with methyl or with iodine reduced activity. Modeling of (+)- and (−)-epibatidine suggested that lipophilic interactions of a nicotinic binding site with the alicyclic ring system might be quite similar, thus accounting for the lack of enantioselectivity. We were also contacted by Ray Baker in May 1993, who provided us with additional (+)- and (−)-epibatidine. In return, we supplied his group with natural *N*-acetylepibatidine and they determined the absolute configuration of natural (−)-epibatidine by comparisons on chiral columns. Epibatidine became an important research tool for the study of nicotinic channels and as a structural starting point for development of nicotinic agents as analgetics, cognitive enhancers, and appetite suppressants and as radioligands for investigation of nicotinic channels. Our own synthetic efforts had been set aside with the offer of synthetic epibatidine and analogues from Corey, but an intermediate in our synthesis, namely methyl 14-carbomethoxytropane-6-carboxylate, was later converted by reaction with the dianion of acetone oxime to epiboxidine, a methylisoxazole analogue of epibatidine, which proved to be more selective than epibatidine for ganglionic nicotinic channels, while being somewhat less potent as an analgetic and much less toxic than epibatidine.[54] Further analogues of epibatidine with methylisoxazole and pyridyl rings have been prepared by our group and are being evaluated for nicotinic activity.

By 1986, we had discovered hundreds of alkaloids in skin extracts of dendrobatid frogs of the genera *Phyllobates*, *Dendrobates*, *Epipedobates*, and *Minyobates*. Some, like batrachotoxin in *Phyllobates* and epibatidine in *Epipedobates*, were found uniquely in a single genus. Most others were more widely distributed, but a few were extremely limited in their distribution. Most of the species of the dendrobatid genera had been examined, and with the exception of three species from the genus *Epipedobates*, all contained significant amounts of alkaloids. During the previous 10 years we had surveyed frogs from more than 50 genera in 11 other families of amphibians and had found no alkaloids. But then almost at the same time, we discovered first pumiliotoxins and allopumiliotoxins in skins of a bufonid toad of the genus *Melanophryniscus* collected by Myers and I in Southeastern Brazil, and then a variety of what we were calling "dendrobatid alkaloids" in skins of two species of mantelline frogs of the genus *Mantella* from Madagascar, which were purchased from a pet dealer, and in skins of two species of myobatrachid frogs of the genus *Pseudophryne* from Australia, which were supplied by a herpetologist, Richard Zweifel. The finding of "dendrobatid alkaloids" in three other families of anurans was reported in 1984,[20] at about the same

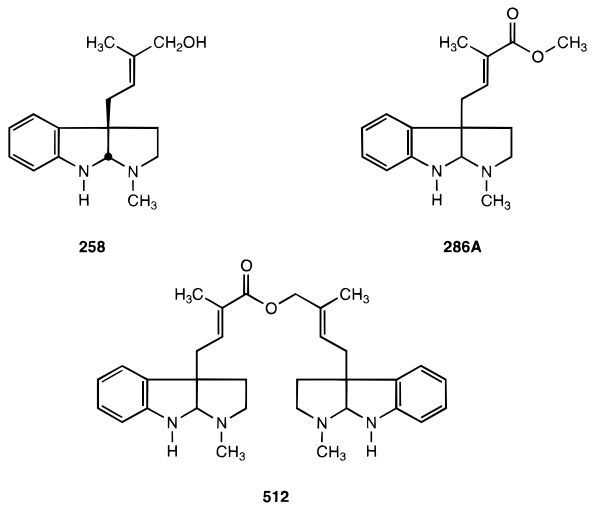


Figure 11. Structures of pseudophrynamines **258**, **286A**, and **512**.

time that the collection of significant numbers of any dendrobatid frogs was curtailed by a CITES listing (see ref 55).

The extracts of skin of *Melanophryniscus moreirae* from Brazil contained major amounts of allopumiliotoxin **323B** (Figure 3) and a pumiliotoxin **267C**, the latter at that time unknown from dendrobatid frogs.[20] Extracts of *Melanophryniscus stelzneri* from Uruguay and Argentina were later provided, respectively, by Vittorio Erspamer and by Eduardo G. Gros. The extracts contained, in addition to pumiliotoxins and allopumiliotoxins, a number of novel homopumiliotoxins and various izidines and decahydroquinolines,[44] many of which had previously been detected in neotropical dendrobatid frogs. The profile of skin alkaloids varied markedly for the two populations of *M. stelzneri*.

The alkaloids from the nocturnal *Pseudophryne* frogs of Australia consisted of pumiliotoxins, allopumiliotoxins, and a new class of indolic alkaloids as yet not detected in any dendrobatid frog. In 1985, Erspamer and his group reported that an alkaloid present in the skin of one population of *Pseudophryne coriacea* from Queensland, Australia, was similar in properties to the pumiliotoxin B, which we had provided to Erspamer, but was much more potent.[56] Examination in our laboratory of fractions proposed by Erspamer to contain the very potent pumiliotoxin revealed pumiliotoxin B as the major alkaloid. It appeared possible that the highly potent pumiliotoxin had degraded to pumiliotoxin B or that a synergistic factor had been lost. To obtain further extracts, a field trip to Queensland and New South Wales was organized. During a 1-month collecting trip, six populations of *P. coriacea* were collected. Extracts were also obtained from several other *Pseudophryne* species. All contained pumiliotoxins and allopumiliotoxins and a new indolic class of alkaloids, the pseudophrynamines.[57] The structures of the pseudophrynamines were deduced from spectral data, and the major three alkaloids are shown in Figure 11. None appeared to be artifacts. Synthesis of pseudophrynaminol **258** in our laboratory by Garraffo[58] allowed biological evaluation. It proved to be a very potent and nonselective blocker of nicotinic channels. The relative amounts of pumiliotoxins/allopumiliotoxins and pseudophrynamines in skin extracts of frogs of the genus *Pseudophryne* varied remarkably even for different populations of the same species.[59] A dietary source for the alkaloids in skin extracts of *Pseudophryne* is unknown, and it should be noted that these frogs are nocturnal rather than diurnal in habit.

The initial results on the alkaloids in skin of Madagascan frogs of the genus *Mantella* obtained through the pet trade[20] were exciting, and contacts were made with Marta Andriantsiferana of the Université d'Antananarivo in Madagascar. In 1989, various species of mantelline frogs were collected with the collaboration of Madagascan students, most notably N. R. Andriamaharavo. Two subsequent collecting trips in 1992 and 1993 were under the sponsorship of the Japanese Council for Culture and Science. Nine of the 11 known species of *Mantella* were collected. The extracts contained a variety of alkaloids, some of which had been previously detected in dendrobatid frogs and some of which had new and novel structures.[45] One major structural group from mantelline frogs were the pumiliotoxins, allopumiliotoxins, and homopumiliotoxins. Apparently, both 8-deoxy and 8-desmethyl analogues were also present. Certain species contained large amounts of 5,8-disubstituted indolizidines and corresponding 1,4-disubstituted quinolizidines. The structure of quinolizidine **217A** (Figure 7) was based on spectral analysis of alkaloid isolated in submilligram amounts from *Mantella baroni*.[60] Certain mantelline species contained the decahydroquinoline **195A** and the homologous hexa- and octahydroquinolines, along with apparent Diels−Alder adducts **384A/B**, presumably derived from hexahydro- and octahydroquinolines of molecular weights 191 and 193 (unpublished results). Thus, the mantelline frogs have proven a source of several unique new alkaloids.

At the present time, about 20 major structural classes of alkaloids have been found in studies that have spanned three decades, three continents, and over 70 genera of frogs/toads from 11 different amphibian families. Frogs of dendrobatid genera *Phyllobates*, *Dendrobates*, and *Epipedobates* have been found to completely lack skin alkaloids when raised in captivity,[25,61,62] as have frogs of the mantelline genus *Mantella*.[63] A variety of environmental manipulations do not trigger alkaloid production, but frogs fed alkaloid-dusted fruit flies efficiently accumulate unchanged such alkaloids exclusively into skin.[61] Certain alkaloids are accumulated more efficiently than others, with pyrrolidines and piperidines being very poorly accumulated. Dietary sources for the pyrrolidines, piperidines, 3,5-disubstituted pyrrolizidines, 3,5-disubstituted indolizidines, 4,6-disubstituted quinolizidines, and 2,5-disubstituted decahydroquinolines found in frog skin now seem to be ants, although it should be noted that the majority of such alkaloids detected in frog skin extracts have as yet not been detected in ants. It is possible that the other classes of izidines and the histrionicotoxins and gephyrotoxins may also prove to be of ant origin. The tricyclic precocinelline is undoubtedly of beetle origin as may be other such tricycles, many of as yet unknown structure. The spiropyrrolizidines, including nitropolyzonamine recently detected in frog skins, and the oximes **236** and congeners will probably prove to be of millipede origin. Although the alkaloid-containing

frogs feed exclusively and selectively on small arthropods, a dietary chain leading to plants cannot be excluded. Indeed, the plant indole alkaloids chimonanthine and calycanthine and the bipyridyl alkaloid noranabasamine were detected in extracts from *Phyllobates terribilis*.[64] Factors, such as differing prey targeting by different frog species, prey availability, and specificity of uptake systems could underlie the marked differences in alkaloid profiles seen in different populations and species of frogs. The specificity of uptake systems apparently is rather well-conserved, on the basis of the rather limited feeding experiments that have been conducted to date with dendrobatid and mantelline frogs. It appears possible that this uptake system is primitive, well-conserved, and merely overexpressed in frogs that accumulate alkaloids. No accumulation of dietary alkaloids occurred with dendrobatid frogs, such as *Colosthethus talamancae*[61] and *Epipedobates azuriventris* (unpublished results), that do not contain detectable skin alkaloids in the wild. A major challenge for the future is to discover the ultimate sources of the highly active batrachotoxins, the cardiotonic pumiliotoxins and homopumiliotoxins, the potent analgetic epibatidine, and the pseudophrynamines.

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